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A Transcriptionally Permissive Epigenetic Landscape at the Vasoactive Intestinal Peptide Receptor-1 Promoter Suggests an Euchromatin Nuclear Position in Murine CD4 T Cells

K.D. Benton¹, R.J. Hermann¹, E.E. Vomhof-DeKrey, J.S. Haring¹, T. Van der Steen¹, J. Smith¹, S. Dovat², and G.P. Dorsam¹

¹Department of Chemistry and Molecular Biology and the Center for Protease Research, North Dakota State University, Fargo, North Dakota, USA

²Division of Hematology-Oncology, Department of Pediatrics, University of Wisconsin-Madison, Madison, Wisconsin, USA

Summary

T cells express receptors for neuropeptides that mediate immunological activities. Vasoactive intestinal peptide receptor – 1 (VPAC1), the prototypical group II G protein coupled receptor, binds two neuropeptides with high affinity, called vasoactive intestinal peptide and pituitary adenylate cyclase activating peptide. During T cell signaling, VPAC1 mRNA expression levels are significantly downregulated through a Src kinase dependent mechanism, thus altering the sensitivity for these neuropeptides during an immune reaction. Presently, it is unknown whether the mechanism that regulates VPAC1 during T cell signaling involves epigenetic changes. Therefore, we hypothesized that the epigenetic landscape consisting of diacetylation at H3K9/14 and trimethylation at H3K4, two transcriptionally permissive histone modifications, would parallel VPAC1 expression showing high enrichment in untreated T cells, but lower enrichment in α -CD3 treated T cells. To this end, quantitative chromatin immunoprecipitation (ChIP) analysis of H3K9/14ac and H3K4me3 was conducted using purified CD4⁺ T cells, with CD45R⁺ B cells as a negative control. Our data revealed that these histone modifications at the VPAC1 promoter did indeed parallel its mRNA levels between T and B lymphocytes, but did not decrease during T cell signaling. Collectively, these data strongly imply a euchromatin nuclear position for the VPAC1 locus irrespective of the activation status of T cells.

Introduction

Bidirectional signaling between the nervous and immune systems is mediated by soluble neuropeptides [1]. Vasoactive intestinal peptide (VIP) is a 28 amino acid protein highly expressed in the central nervous system and is delivered to immune organs by the peripheral nervous system [2]. VIP binds a seven transmembrane, group II, G protein coupled receptor belonging to the glucagon/secretin family termed vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide receptor -1 (VPAC1) [3]. Immune cells expressing VPAC1, and in close proximity to VIPergic nerves, respond to VIP, which in turn modulate numerous cellular activities [4]. For example, in the innate immune system, VIP/VPAC1

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signaling has been shown to be a potent macrophage deactivating factor [5,6]. An effect of VIP in the adaptive immune system is the inhibition of T cell proliferation by suppressing IL-2 production [7].

Murine T cells express high levels of VPAC1 [8], whereas B cells show undetectable VPAC1 expression [9,10]. Anti-CD3-treated CD4⁺ T cells express VPAC1 nearly ten-fold less compared to untreated cells [11,12]. Recently, we published pharmacological evidence showing that the Src kinases, Fyn and Lck, are essential for downregulating VPAC1 steady-state levels during CD4⁺ T cell signaling [8]. It is unclear, however, whether this downregulation in VPAC1 message is accompanied with changes in its chromatin state. Therefore, additional research is warranted to investigate the gene regulatory mechanisms of VPAC1 expression during T cell signaling.

A major mechanism that regulates gene expression is the accessibility of DNA regulatory elements [13]. DNA is packaged in eukaryotic cells as chromatin, which consists of a protein complex with 4 unique histone dimers called H2A, H2B, H3, and H4, that is “spooled” around itself by 147 bp of DNA [14]. This histone/DNA complex forms the repeating chromatin element called the nucleosome, whose spherical structure has been determined by X-ray crystallography to 2.8 Å [15]. Protruding out from the nucleosome circumference are the cationic histone N-terminal domains called “histone tails” that can be post translationally modified, including acetylation and methylation [16]. Histone acetylation at H3K9 and H3K14 [17], and trimethylation at H3K4 immediately upstream of type II promoters correlates extremely well with transcriptional activation [18-20]. We hypothesized that differential expression of VPAC1 in CD4 T cells treated in the presence and absence of anti-CD3 would parallel the enrichment levels of the transcriptionally permissive H3K9/K14ac and H3K4-me3 modifications at the VPAC1 promoter.

To test our hypothesis, snapshots of the VPAC1 promoter were conducted by quantitative chromatin immunoprecipitation (ChIP) using primary murine CD4⁺ T cells, with CD45⁺ B cells as a negative control. In support of our hypothesis, the expression differences between CD45R⁺ B and CD4⁺ T cells correlated well with the levels of H3K9/K14ac and H3K4-me3 at the VPAC1 promoter. Surprisingly, H3K9/K14ac and H3K4me3 were not decreased as hypothesized during T cell signaling supporting two major conclusions. First, the chromatin state of VPAC1 enriched for H3K9/14ac and H3K4me3 cannot explain the downregulation of VPAC1 during T cell activation, and second, the VPAC1 locus remains associated with euchromatin irrespective of TCR signaling.

Materials and Methods

Reagents

Trypan Blue and 1x PBS without Ca²⁺ and Mg²⁺ were purchased from Cellgrow. Glycine and SDS were purchased from Fisher Scientific. EDTA, dNTPs, GoTaq Green Master Mix, ethidium bromide, and M-MLV Reverse Transcriptase were obtained from Promega Corporation. Protein A-agarose/salmon sperm DNA and anti-acetyl-Histone H3 (Lys9/14), anti-acetyl-Histone H3 (Lys9), anti-acetyl Histone H3 (Lys14), and EZ-ChIP kit were obtained from Upstate and anti-trimethyl H3 (Lys4) was obtained from Abcam. Anti-CD4 and anti-CD45R labeled magnetic beads were obtained from Miltenyi Biotec. Protease Cocktail Inhibitors were purchased from Calbiochem. RNase A was obtained from Novagen and Proteinase K was purchased from Ambion. SYBR green master mix and Taqman master mix were obtained from Applied Biosystems. The following antibodies (and clones) were obtained from Biolegend: anti-CD4-FITC (RM4-4), anti-CD4-FITC-PE-Cy5 (G1C1.5), anti-CD25-PE (3C7), anti-CD43-PE (1B1), anti-CD44-PE (IM7), anti-CD127-PE (1L-7Ra), anti-CD62L-PE

(Mel-14), anti-CD117-PE (ACK2), anti-CD44-FITC (IM7), anti-CD69-PE (H1,2F3) and anti-CD45R-FITC (RA3-6B2). All other reagents were obtained from Sigma or VWR.

Lymphocyte isolation, α -CD3 treatment of CD4⁺ T cells and antibody staining

CD4⁺ T cells were isolated to a purity of $\geq 93\%$ and treated with α -CD3 as previously described [8]. CD45R⁺ B cell populations were isolated from CD4⁺ T cell depleted non-adherent splenocytes. Trypan blue stained cells (0.2%) were counted using a hemocytometer to determine cell number and confirm high viability ($\geq 90\%$). Cells were centrifuged at 500 x g for five minutes, resuspended in 93 μ L PBS/0.5% BSA and 7 μ L anti-CD45R labeled magnetic beads, and refrigerated for 20 minutes. B cells were purified using the positive selection option and passed through a magnetic column twice on a Miltenyi Auto-MACs instrument (Auburn, CA) as described by the manufacturer ($\geq 97\%$). To confirm a naïve B cell phenotype isolated CD45R⁺ B cells were co-stained with α -CD45R-FITC and α -CD43-PE, and sorted by flow cytometry (FACSCalibur; San Jose, CA, or Accuri C6; Ann Arbor, MI) as described previously [8].

Chromatin Immunoprecipitation

Assays were performed according to the EZ-ChIP kit protocol (Millipore, Bellerica, MA). Briefly, 10×10^6 CD4⁺ T cells (+/- 1 μ g/ml plate bound α -CD3) or CD45R⁺ B cells were cross-linked with 1% formaldehyde for ten minutes at room temperature, followed by quenching with 0.125 M glycine for five minutes. Cells were washed with ice cold PBS, centrifuged at 500 x g for five minutes, resuspended in SDS lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, protease cocktail inhibitors) and sonicated nine times for thirty seconds at 40% duty with an output of 4 followed by a one minute incubation on wet ice between sonications (Branson Sonifier, Danbury, CT). Samples were pre-cleared with protein A-agarose/salmon sperm DNA followed by incubation with 2 μ g of antibody of interest overnight with gentle rotation at 4°C. Protein A agarose/antibody/protein/DNA complexes were sequentially washed for 5 minutes at 4°C in low salt buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100), high salt buffer (20 mM Tris, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100), LiCl buffer (10 mM Tris, 250 mM LiCl, 1 mM EDTA, 1% deoxycholate, and 1% Nonidet P-40), and 2 washes of TE buffer (20 mM Tris and 2 mM EDTA). DNA was collected from the complexes with elution buffer (1% SDS and 100 mM NaHCO₃), reversal of the cross-links in 0.2 M NaCl at 65°C, sequential treatment with RNase A and Proteinase K, and phenol-chloroform extracted followed by ethanol precipitation. Reconstituted immunoprecipitated DNA samples in water were used immediately in semi-quantitative PCR or quantitative real time PCR or frozen at -20°C until assayed.

Production of Taq DNA Polymerase

Recombinant *E. coli* clone, DSB1-alpha-1, carrying the plasmid pTaq was a kind gift from Dr. Phil McClean at North Dakota State University. Cells were incubated at 37°C in 50 mL of 2X-YT broth (16g tryptone, 10g yeast extract, 5g NaCl) supplemented with 100 μ g/mL ampicillin overnight until OD₅₉₀ of 0.6-0.8 was reached. Cells were subcultured in 500 mL of 2X-YT broth until mid-log phase (OD₅₉₀ = 0.8) was reached, approximately 3-4 hours. Expression of Taq polymerase was induced by 0.5 mM isopropyl- β -D-thiogalactoside. Post-induction (17 hours), cells were centrifuged at 4000 x g at 4°C for 5 minutes. Pellets were washed once with STE (0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0), centrifuged as above, resuspended 1:1 (v/v) with 3 mL of STE and 3 mL of 4x storage buffer (200 mM Tris-HCl, pH 8.0, 400 mM NaCl, 0.4 mM EDTA, 2.0 mM dithiothreitol and 4.0% Triton X-100). Cells were incubated at 75°C for 1 hour with periodic mixing, followed by sonication with a Branson sonifier set at 40% duty cycle for 2 minutes. Cell lysates were centrifuged for 10 minutes at 12,000 x g at 4°C

C to remove denatured proteins and cellular debris. Supernatants were diluted with an equal volume of glycerol and stored at -20°C with little enzymatic activity loss over 24 months.

Semi-quantitative PCR

Immunoprecipitated DNA templates obtained from ChIP were amplified with partially purified 0.4 μ L recombinant Taq (1.7 mg/ml stock concentration), 200 μ M dNTP, 1x GoTaq Green Master Mix, and 2 μ M primers in a total volume of 20 μ L. Amplification proceeded with 32-36 repeating cycles of 94°C for 15 seconds, 60°C for primer sets 2-5 and 67°C for primer set 1 for 15 seconds, and 72°C for 15 seconds. PCR amplification products were separated on a 3% ethidium bromide stained, agarose gel and visualized using a Syngene digital camera (Frederick, MD).

SYBR Green qPCR

Quantative PCR was performed according to the manufacturer's protocol (ABI, Foster City, CA). Briefly, final concentrations of reactions were, 1x SYBR Green, 0.5 μ M primer set 2, 1/2.5 dilution of IP DNA template (10 μ L) in a final reaction volume of 25 μ L. Samples were amplified for 40 cycles in duplicates. Three serial dilutions of DNA template were used and a Δ Ct normalized to input within 50% were used for data analysis. Reactions were carried out on a 7500 Real Time PCR System (Applied Biosystems) and relative fold enrichment was determined using the $\Delta\Delta$ Ct method described previously [11].

cDNA generation and Taqman PCR (qPCR)

Taqman qPCR was performed as previously described [11]. Briefly, total RNA (2 μ g) was used to generate first strand cDNA using M-MLV reverse transcriptase according to the manufacturer's protocol. Final concentration in a real time PCR reaction containing 1X ABI master mix, 0.2 μ M of a 5' labeled 6 carboxyfluorescein (FAM) and 3'-labeled quencher dye 6-carboxytetramethylrhodamine (TAMRA) labeled probe (mVPAC1, Accession No. Nm_011703, 1218-1242 bp, 5'-TCCCCGACAACCTTTAAGGCCAGGT-TAMRA-3'; mHPRT, Accession No. Nm_013556, 659-687 bp, 5'-TGTTGGATACAGGCCAGACTTTGTTGGAT-TAMRA-3') and 300 nM of each primer (mVPAC1, 1188-1212 bp, 5' primer TTGGAGTTCACATGTGTCATGTTTC, mVPAC1, 1245-1268 bp, 3' primer CTACGACGAGTTCAAAGACCATT; mHPRT, 636-655 bp, 5' primer CTGGTGAAAAGGACCTCTCG, mHPRT, 719-744 bp, 3' primer TGAAGTACTCATTATAGTCAAGGGCA). Primer and probe sequences were designed as previously described. PCR reaction conditions were 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Results

Presence of transcriptionally permissive epigenetic signals at the VPAC1 promoter in lymphocytes

The main objective of this study was to investigate the epigenetic landscape of the VPAC1 promoter in primary, murine T cells. The first question we asked was whether the VPAC1 promoter in CD4 T cells contained transcriptionally permissive histone acetylation and methylation modifications. To this end, the VPAC1 promoter was segmented into 5 regions of approximately 200 bp (Fig. 1A) and sequence specific primers were synthesized for PCR amplification (Table 1). Chromatin immunoprecipitation (ChIP) was performed using crosslinked CD4 T cells and chromatin was successfully sheared into a gaussian distribution providing on average chromatin segments of approximately 500 bp or 2 nucleosomes (Data not shown). Results from semi-quantitative PCR showed substantial enrichment for H3K9/K14ac and H3K4me3 over IgG control (Fig. 1B). To distinguish between acetylation at K9

verses K14, antibodies to these residues were also employed demonstrating similar enrichment for both H3K9ac and H3K14ac, respectively. These ChIP data confirmed the presence of transcriptionally permissive epigenetic signals at the VPAC1 promoter that was constant throughout or gradually increased from -1000 bp to the transcriptional start site (Fig. 1C). Collectively, the profile of H3K9/14ac and H3K4me3 at the VPAC1 promoter correlated well with the high expression levels for VPAC1 in primary CD4 T cells.

Acetylation at H3K9/14 and trimethylation at H3K4 correlates with VPAC1 expression levels between murine T and B cells

In mouse, VPAC1 steady state mRNA levels are high in T cells but low/undetectable in B cells [8-10]. Shown in fig. 2A is typical purity of separated CD4⁺ T cells and CD45⁺ B cells by flow cytometry ($\geq 93\%$ and 97% , respectively). These separated cells revealed more than 50-fold higher VPAC1 levels in CD4⁺ T cells compared to CD45⁺ B cells by qPCR (Fig. 2B). Therefore, we hypothesized that B cells should have significantly less if any H3K9/14ac and H3K4me3 modifications as assessed by ChIP. We employed semi- and quantitative PCR (Taqman) using primer set 2 as representative of the VPAC1 promoter to assess immunoprecipitated (IP) chromatin samples for H3K9/14ac and H3K4me3 enrichment. As expected, figures 2C and 2D showed significantly more enrichment for these modifications in T cells versus B cells. To test for specificity of the antibodies used, primers specific for an intergenic region upstream (108 Kb) from the VPAC1 locus showed undetectable levels for either modifications (Fig. 2C; negative control). We conclude that the epigenetic landscape at the VPAC1 promoter, regarding diacetylation at H3K9/K14 and trimethylation at H3K4me3, parallels its high expression levels in T cells and low/undetectable levels in B cells.

Downregulation of VPAC1 during T cell signaling is not mediated by changes in H3K9/14ac or H3K4me3 levels

Mouse and human steady-state VPAC1 mRNA levels are downregulated during T cell signaling and we previously published pharmacological evidence showing Src and JNK kinase inhibitors blocking this effect [8]. In addition, JNK1 activity has been reported to alter histone acetylation levels [21,22]. Consequently, we hypothesized that VPAC1 downregulation during T cell signaling was primarily regulated at the transcriptional level by a decrease in the histone enrichment of H3K9/14ac and H3K4me3. Therefore, we treated CD4⁺ T cells +/- α -CD3 for 24 hr and confirmed T cell signaling by measuring early activation markers (CD69 and CD127) by flow cytometry, and the downregulation in VPAC1 expression by qPCR. As expected, there was a significant increase in CD69 expression (3.5% vs. 40.3%) and a large decline in CD127 expression as assessed by flow cytometry (Fig. 3A). Also, Fig. 3B illustrates that VPAC1 levels went down by 70% in cells treated with α -CD3 as we have published previously [11,23]. Surprisingly, quantitative ChIP analysis revealed no demonstrable change in H3K9/14ac and H3K4me3 after 24 hours with α -CD3 (Fig. 3C and 3D). These data strongly support that the mechanism regulating downregulation of VPAC1 expression during T cell activation cannot be explained by a decrease in these transcriptionally permissive epigenetic signals. Furthermore, these findings support a euchromatin nuclear position for the VPAC1 locus irrespective of the activation status of T cells.

The transcriptionally permissive chromatin state at the VPAC1 promoter in B cells supports its expression

Originally, murine B cells were chosen as a negative control as VPAC1 had previously been undetectable in these cells by semi-quantitative PCR [9,10]. However, the enrichment of the two transcriptionally permissive histone modifications detected in B cells, albeit lower than in T cells, was enriched over non-specific IgG control. Therefore, in an attempt to further substantiate the histone code as an accurate predictor of active gene expression, we

quantitatively measured VPAC1 mRNA levels in sorted naïve T and B cells by qPCR; a more sensitive technique than previously used [9,10]. Highly purified naïve T cells (CD4⁺/CD25⁻/CD44⁻) showed two-fold greater VPAC1 levels by qPCR confirming high levels of this GPCR in the naïve T cell pool (Fig. 4B). Importantly, further characterization of CD45R⁺ B cells with CD43 expression by flow cytometry showed no detectable VPAC1 levels in naïve CD45R⁺/CD43⁻ B cells by qPCR. Whereas, the most predominant VPAC1 expression was observed in the CD45R⁺/CD43⁺ B cell pool (Fig. 4C and 4D). These results suggest that the low enrichment of H3K9/14ac and H3K4me3 measured in CD45R⁺ B cells is most likely derived from the CD45R⁺/CD43⁺ population rather than the naïve B cell population. Furthermore, to our knowledge, this is the first quantitative observation of VPAC1 expression in CD45R⁺/CD43⁺ B cells, which may represent a pre-B cell development stage, an activated B cell population or other hematopoietic cell type [24,25]. As VIP has been shown to alter isotype switching in human B cells [26], this observation may indicate that VPAC1 expression is inducible during B cell activation and signaling from this GPCR could contribute to tailoring of the F_c region of the antibody pool. Additional research is needed to further characterize this CD45R⁺/CD43⁺ B cell population.

Discussion

This is the first quantitative analysis of the chromatin profile of the VPAC1 receptor using murine lymphocytes [9,10]. H3K9/14ac and H3K4me3 profiles suggest an open and transcriptionally active chromatin state consistent with a euchromatin subnuclear position of the VPAC1 locus in T and B cells [17,27,28]. The magnitude of H3K9/14ac and H3K4-me3 enrichment likewise correlated well with VPAC1 expression levels between T and B lymphocytes. In contrast, these transcriptionally active histone modifications did not significantly change during T cell activation suggesting additional mechanisms controlling VPAC1 downregulation other than removal of H3K9/14ac and H3K4-me3 modifications. Lastly, to our knowledge, this is the first quantitative measurement of VPAC1 steady-state mRNA levels in murine CD45R⁺/CD43⁺ B cells.

VPAC1 expression in murine B cells has failed to show detectable VPAC1 expression using semi-quantitative PCR technology [9,10]. In agreement to these studies, our quantitative qPCR measurements showed no detectable VPAC1 mRNA levels in naïve CD45R⁺/CD43⁻ B cells (Fig. 4D). We purport that enriched H3K9/14ac and H3K4-me3 at the VPAC1 promoter observed in this study was due to co-purified CD45R⁺/CD43⁺ hematopoietic cells. If we assume that this CD45R⁺/CD43⁺ population represents activated B cells, it may suggest that plasma B cells are directly sensitive to VIP/PACAP. For example, VIP has been shown to induce IgA isotype switching in human B cells [26,29,30]. Nonetheless, VPAC1 expression in CD45R⁺ B cells was shown to be 60 fold less than CD4⁺ T cell levels. Such disparate expression between two cells derived from a common haemopoietic precursor may be explained by unique external cues within their respective niches during T cell maturation (thymus) and B cell development (bone marrow; Fig. 5A). Additional research is needed to further characterize this CD45R⁺/CD43⁺ B cell population.

Histone diacetylation at H3K9/K14 has been shown to represent euchromatin domains [31]. Our quantitative ChIP data showing similar levels of H3K9/14ac at the VPAC1 promoter, irrespective of the activation status of the T cell, would support a euchromatin architecture. Furthermore, these data indicate that the VPAC1 locus does not appear to possess a heterochromatin state and strengthens the notion that the mechanism controlling VPAC1 downregulation in α -CD3 treated T cells is not due to a shift from a euchromatin to a heterochromatin state. There is strong evidence demonstrating that methylation at H3K9, which is mutually exclusive with acetylation at H3K9, has been correlated with gene silencing, as it recruits heterochromatin protein 1 (HP1) resulting in a heterochromatin-like environment

[32]. Thus, the maintenance of high levels of H3K9/14ac at the VPAC1 promoter during T cell signaling promotes a euchromatin state, but does not seem to play a direct role in VPAC1 downregulation. Figure 5B summarizes our working model regarding possible additional mechanisms regulating the downregulation of steady-state VPAC1 mRNA levels during T cell activation. These mechanisms include: i.) repressive transacting factors binding to the accessible VPAC1 promoter ii.) changes in mRNA stability or iii.) increases in repressive epigenetic signals.

With respect to trans-acting factors, we reported that the repressive, anti-leukemic factor, Ikaros, downregulated VPAC1 when ectopically expressed in mouse fibroblasts [33]. The main conclusion was that Ikaros engaged the VPAC1 promoter at Ikaros binding motifs and directly suppressed transcription. In 2007, we showed *in vivo* ChIP data supporting promoter occupancy by Ikaros in activated human CD4 T cells engaging a cluster of four high-affinity Ikaros binding elements [34]. These studies may suggest that the open promoter state allows for Ikaros recruitment and subsequent gene suppression of VPAC1. Ikaros binds and recruits a number of co-repressors including nucleosome remodeling and deacetylation complex (NuRD), CtBP and CtIP [35-39]. That NuRD cannot bind H3K4me3 modified histones may further suggest that the mode by which Ikaros downregulates VPAC1 expression might be through a NuRD-independent manner [40]. In toto, these data support a trans-acting mechanism (Ikaros) responsible for shutting down the transcriptional rate of VPAC1 during T cell signaling. Additional important questions remain regarding the mechanism for how Ikaros regulates VPAC1 expression during T cell activation.

In contrast to trans-acting factors, high levels of transcriptionally active histone modifications, H3K9/14ac and H3K4me3, could suggest that the transcriptional rate of VPAC1 does not change during T cell activation. If this were the mechanism, then Src and JNK kinases, downstream of the TCR, would downregulate steady-state VPAC1 mRNA levels by a post-transcriptional mechanism. There are two major justifications for hypothesizing this mechanism. First, global histone acetylation levels are evolutionarily conserved from *drosophila* to human, and correlate with gene expression and high transcriptional rates [41, 42]. Second, H3K4me3 has been linked with active transcription as components of the spliceosome and chromatin helicase DNA binding protein 1 (CHD1) bind H3K4me3 modified histones. CHD1 contains a chromodomain and interacts with components of the spliceosome, thus linking this signal to transcription and mRNA splicing [20,43]. Collectively, we propose that a decrease in mRNA stability (by a non-coding RNA species for example) rather than transcriptional rate may explain the downregulation of VPAC1 steady-state mRNA levels during T cell activation. In order to substantiate this conclusion, experiments are ongoing to directly measure the transcriptional rate and mRNA half-life for VPAC1 in activated CD4 T cells.

The final major mechanism that could mediate VPAC1 downregulation during T cell activation could be repressive histone modifications at the VPAC1 promoter. An elegant study by Susan Chan's group clearly showed that well-established transcriptionally permissive histone modifications (H3K9/14ac, H3K4me2 and H3K4me3) all remained high at the Hes-1 promoter in developing murine thymocytes, but was instead heritably silenced by a repressive H3K27me3 mark [44]. This report underscores the importance to evaluate the epigenetic landscape of any gene independently. It may also expose clear deficiencies with respect to a "histone code", which may more aptly be described as an "epigenetic language" [45].

Establishing the molecular interplay between the nervous and immune systems will be an important component to understanding mechanisms controlling neuroimmunomodulation. A critical biochemical link between these two systems is the expression of the prototypical class II GPCR, VPAC1, in lymphocytes [46]. The current study demonstrates that the epigenetic

landscape of the promoter for VPAC1 in CD4⁺ T cells is consistent with an euchromatin state, and H3K9/14ac and H3K4me3 modifications may have a greater regulatory influence for VPAC1 in the absence of TCR signaling. Lastly, the transcriptionally permissive histone modifications found at the VPAC1 promoter were further validated by quantitatively confirming VPAC1 expression in CD45R⁺/CD43⁺ but not CD45R⁺/CD43⁻ B cells. Future studies investigating the molecular mechanism controlling VPAC1 regulation in naïve and activated hematopoietic cells is essential to better understanding its role in infectious disease and autoimmunity.

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A. **B.**

C.

Figure 1. The mouse VPAC1 promoter in CD4 T cells is enriched for H3K9/14ac and H3K4me3
A. Schematic representation of the VPAC1 5' flanking region (-1000 to +1 bp; horizontal bar) with an arrow indicating the transcriptional start site. This region was segmented into five $\approx$ 200 bp regions named 1-5 and demarcated by lines underneath the promoter. The dotted line represents the minimal VPAC1 promoter. **B.** Splenic, naïve CD4⁺ T cells were analyzed by ChIP analysis for enrichment of H3K9/K14ac (mono- and di-acetylated), and H3K4me3 histone signals as indicated. Numbers to the left indicate the primer set used for semi-quantitative PCR amplification. Gels were separated on an ethidium bromide stained 3% agarose gel and visualized by a digital CCD camera. Data is representative of three independent experiments. **C.** Densitometry from diacetylated H3K9/14 and H3K4me3 enrichment at the VPAC1 promoter obtained in **C**. Square, IgG; Triangle, diacetylated H3K9/K14; circle, H3K4me3.

A. **B.**

Figure 2. Differential enrichment of H3K9/14ac and H3K4me3 histone modifications at the VPAC1 promoter

A. Flow cytometric analysis of mouse CD4⁺ T cells and CD45R⁺ B cells. *Left Panel:* Non-adherent splenocytes were stained with anti-CD4-FITC pre- (red line) and post- (blue line) CD4 positive magnetic bead separation (*Materials and Methods*) with typical purities $\geq 93\%$ (n=3). *Right Panel:* CD4 depleted non-adherent splenocytes were subsequently stained pre- (red line) and post- (blue line) CD45R-FITC positive magnetic bead separation (*Materials and Methods*) with typical purities $\geq 97\%$ (n=3). **B.** Bar graph for Taqman qPCR measurements for VPAC1 from indicated cell type. A hash mark indicates a shift in the y-axis. Data is represented as means $\pm$ SEM for relative VPAC1 levels normalized to HPRT and calculated by $2^{(-\Delta Ct)}$

formula from three independent experiments. The asterisk (*) represents a $p \leq 0.05$ as compared to $CD4^+$ T cells. **C-D.** ChIP analysis was performed using purified $CD4^+$ T and $CD45R^+$ B cells followed by quantitative SYBR green PCR using VPAC1 primer set 2 or genomic negative control. **C.** Amplification reactions from SYBR green qPCR using primer set 2 were separated on a 3% agarose gel, stained with ethidium bromide and visualized by a CCD camera (Syngene). This experiment was repeated twice with similar results. **D.** Bar graph showing fold-enrichment over IgG control of H3K9/14ac and H3K4me3 from the indicated cell types. Cycle thresholds (Ct) were subtracted from input Ct values to obtain relative ΔCt values. Fold increases were calculated by $2^{-(\Delta Ct)}$ formula with non specific IgG levels normalized to input arbitrarily set to 1 (not shown). Data is presented as means $\pm$ SEM from three biologically independent experiments (*, $p \leq 0.05$).

Figure 3. Transcriptionally permissive modifications, H3K9/K14ac and H3K4me3, are not removed during T cell signaling

ChIP analysis was performed using purified CD4⁺ T cells incubated +/- plate bound α -CD3 (*Materials and Methods*) followed by quantitative SYBR green PCR using VPAC1 primer set 2. All data is from three independent biological experiments. **A.** Flow cytometric analysis of early activation markers, CD69 (left two panels, increase expected) and CD127 (right far panel, decrease expected) using CD4⁺ T cells treated +/- anti-CD3. **B.** Bar graph representing relative VPAC1 expression levels in CD4⁺ T cells treated +/- anti-CD3 as indicated. Data is presented as means +/- SEM and an * represents a $p \leq 0.05$ as compared to cells without anti-CD3. **C.** Semi-quantitative amplification reactions using immunoprecipitated chromatin and primer set 2 were separated on a 3% agarose gel, stained with ethidium bromide, and visualized by a CCD camera (Syngene). **D.** Bar graph of fold enrichment over IgG control of H3K9/14ac and H3K4me3 from the indicated cell types. Cycle thresholds (Cts) were subtracted from input Ct values to normalize to input and fold increases were calculated by $2^{-(\Delta C_t)}$ formula. Non-specific IgG levels were also normalized to input and arbitrarily set to 1 (not shown). Data is presented as means +/- SEM.

Figure 4. VPAC1 is highly expressed in naïve T cells but not naïve B cells

A. Mouse non-adherent splenocytes were stained with anti-CD4-PE/Cy5, anti-CD25-PE and anti-CD44-FITC. CD4⁺/CD25⁻/CD44⁻ T cells were sorted by flow cytometry. **B.** CD4⁺/CD25⁻/CD44⁻ T cells were used for total RNA isolation, cDNA synthesis and used for qPCR (*Materials and Methods*). Bar graph representing VPAC1 mRNA expression levels measured by qPCR from the indicated T cell populations. Data is graphed as means $\pm$ SEM from three independent biological experiments. **C.** Mouse non-adherent splenocytes were stained with anti-CD45R-FITC and anti-CD43-PE. CD45R⁺/CD43⁻ B cells were sorted by flow cytometry. **D.** CD45R⁺/CD43⁻ B cells were used for total RNA isolation, cDNA synthesis and used for qPCR (*Materials and Methods*). Scatter plot representing VPAC1 mRNA expression levels measured by qPCR from the indicated T cell populations is shown. Means from duplicate replicates (circles) from two or three independent biological experiments (means are

represented as horizontal lines) is presented. Samples that showed no VPAC1 detection is labeled N.D. standing for not detected.

Figure 5. Working model of VPAC1 regulatory mechanism

A. Schematic representation of the epigenetic state at the VPAC1 promoter between T and B cells generated from a common haematopoietic stem cell (HSC). The font size of ac or me3 indicates the extent of enrichment of these modifications as determined from this study. The black arrow size at the transcriptional start site (TSS) indicates the transcriptional rate. **B.** TCR signaling in CD4 T cells decreases VPAC1 steady-state mRNA expression levels through a Src→JNK kinase dependant mechanism [8,11]. Possible mechanisms by which TCR signaling downregulates VPAC1 steady-state mRNA levels are shown. Change in chromatin state is crossed out as H3K9/14ac and H3K4me3 levels remain unchanged, suggesting that the VPAC1 promoter remains in euchromatin, and cannot explain such regulation. Question marks indicate those mechanisms that cannot be ruled out and are major future goals of our laboratory.

Table One

VPAC1 Promoter Primers for ChIP

Primer	Forward	Reverse
1	5'-CCGGGGTTGAGAGAGAAAGGTGCG-3'	5'-TGACGGGCGGCGGGGTTAGCCAAT-3'
2	5'-CTCGGCTTCTCCCTTGTCGAGAT-3'	5'-GCCCGCACCTTTCTCTCTCAACC-3'
3	5'-CAAGAAAGGGTACAGGCTTTGGTC-3'	5'-GGAACCATCTGCGACAAGGGAGAA-3'
4	5'-AGCAGCCTCAGATCACCCGCTA-3'	5'-GCTCGTGAGGGACGAGAAGTCAAT-3'
5	5'-TCCGCCCCTCCTCCACCTTCTAT-3'	5'-AGCTTGTCCTTAGGGCTCGGATTA-3'
Heterochromatin Region (Negative Control)	5'-GAGTTTATCAGAGCGACTGAACG-3'	5'-AATTGTGGTATCAGTGGGTCATC-3'